Annals of Clinical and Analytical Medicine
Original Research

The effect of Urtica Urens on A549 lung cancer cell line

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Abstract
Aim: Urtica Urens is expected to produce effective results in reducing cancer cell growth as it exhibits anti-oxidant properties. The present study aims to investigate the effect of Urtica Urens on A549 lung cancer cell line.
Material and Methods: Leaves of Urtica Urens were obtained from a local market and its freeze-dried mixture was stored at -20ºC. A549 lung cancer cells were incubated with either 5 or 10 µg/ml of aqueous extract of dried leaves or freeze-dried leaves preparations of Urtica Urens for 24 hours. The obtained data were analyzed using Graph Pad Prism software version 6.
Results: The freeze-dried Urtica Urens showed a significant rise in late apoptosis phase and necrosis, respectively, as compared to the use of 5 µg/ml of aqueous extract of dried and the freeze-dried Urtica Urens. The cells were arresting either in G0/G1 phase (in freeze-dried leaves extract) or in G2/M phase (in dried leaves extract), under 10µg/ml concentration.
Discussion: Urtica Urens was significantly producing more cellular and mitochondrial ROS, as compared to the untreated A549 cells control. The study concluded that cytotoxic effects are produced by dried extracts of Urtica Urens leaves on cancer cell lines, under certain concentrations.

Keywords
Apoptosis; Cell line; Necrosis; ROS

DOI: 10.4328/ACAM.20133 Received: 2020-02-12 Accepted: 2020-03-17 Published Online: 2020-03-30 Printed: 2020-09-01 Ann Clin Anal Med 2020;11(5):510-514
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Introduction

Cancer is known as an important health problem across the world. In 2008, 13% (7.6 million) of all the deaths occurred because of cancer and this number is likely to rise over 11 million in the year 2030 [1]. The most prevalent type of cancers includes lung cancer, breast cancer, and colorectal cancer. The prevalence of lung cancers has increased in developing countries because of its increased incidence, rapid progression, and poor prognosis [2,3]. Lung cancer has emerged as a life-threatening disease and it is the most significant type of cancer that increases mortality rate across the world [4]. There is a 5-year survival rate of 15% among the patients suffering from lung cancer. However, the risk of developing lung cancer or increased mortality rates associated with lung cancer can be prevented through timely detection, diet and food supplements. Recently, different types of cancers have been treated effectively through chemotherapy procedure; however, there is a wide range of side effects associated with the anti-tumor chemotherapies. Therefore, the researchers are exploring an alternative treatment for treating cancer with minimum or no side effects [2]. Cancer is considered an aggressive killerdespite considerable efforts exerted to minimize its occurrence and produce an effective treatment. A considerable amount of cost has been invested for developing the novel synthetic chemotherapeutic agents; however, its clinical use has failed to fulfill the desired expectations during the last decade. This increases the demand for developing new, affordable, and effective anti-cancer drugs [5].

On the contrary, natural remedies along with the medicinal plants have been used for ages to treat different diseases as they have the capability to cure a wide range of illnesses [6].

The chemical compounds derived from plants are extensively used for treating human illnesses since the emergence of ancient medicine [7].

Previous studies have shown that naturally derived therapeutic drugs having the potential to treat cancer have received increased attention over the past 30 years [8,9]. Underlying the significance of these therapeutic compounds, the use of plant-derived products inhibiting various stages of tumorigenesis and associated inflammatory processes has become evident.

The majority of the drugs i.e. 60% of the drugs isolated from the natural products are used for treating cancer, currently [10]. The plants used for deriving these natural products include Camptotheca alkaloids, Podophyllum lignans, Taxus diterpenes, and vinca alkaloids [7].

A study conducted by Saklani and Kutty [11] tested the effectiveness of 16 new plant-derived compounds in treating cancer. The results demonstrated that meisoindigo, isolated from the Chinese plant Indigofera tinctoria and flavopiridol, isolated from the Indian tree Dysoxylum binectariferum produced effective results for treating cancer with lesser side effects and level of toxicity. Studies have also demonstrated the significance of medicinal plants that are used as an alternative for treating cancers in many countries [13,13]. A total of 3000 plants account for exhibiting anti-cancer properties [7].

It has been shown that there are certain compounds derived from plants are used as anti-cancer agents [6,14].

For instance, Urtica Urens, also known as small nettle and belonging to genus Urtica is a semi-woody plant that grows annually. Previously, it was reported that the herb Urtica Urens is used for treating skin inflammations and fever [6].

Flavonoids, caffeic acid, sitosterol, caffeoyl-esters, scopoletin, fatty acids, minerals, and polysaccharides are known as the main constituents of Urtica Urens. It is anticipated that Urtica Urens may produce effective results in reducing cancer cell growth because it consists of flavonoids and other compounds exhibiting antioxidant properties. The pharmacological data supporting the effective use of Urtica Urens is lacking, despite its widespread use in traditional plant medicines. Therefore, the present study aims to investigate the effect of Urtica Urens on A549 lung cancer cell line. The study holds significance as it is the first study investigating the effectiveness of Urtica Urens on A549 lung cancer cell line. In clinical practice, the study results would be helpful for the oncologists as they are exploring an alternative treatment for treating cancer with minimum or no side effects, as compared to the chemotherapeutic drugs.

Material and Methods

Chemicals

The chemicals used in this study for tissue culture, detecting cytotoxic assay, detecting cells cycle assay, and determining reactive oxygen species production assays have been listed below.

- Tissue culture: high glucose Dulbecco's Modified Eagle's Medium powder (DMEM) (Hyclone, USA), foetal bovine serum (FBS) (Hyclone, USA), L-glutamine (Sigma-Aldrich Chemical Co, UK), trypsin/EDTA (Gibco, Canada), antibiotic (penicillin/streptomycin) (Gibco, Canada), and non-essential amino acids (NEAA) (Gibco, Canada).
- Detection of cytotoxicity assay: 3-(4, 5–dimethylthiazol-2-yl) and 2,5- diphenyltetrazolium Bromide (MTT) (Sigma-Aldrich chemical Co, UK).
- Detection of cell cycle arrest: Phosphate buffered saline (PBS) tablets (Oxoid, UK) and the RNase (Abcam, UK).
- Detection of cell death mechanism assays: propidium iodide (PI) (Sigma-Aldrich Chemical Co, UK) and Annexin V-FITC apoptosis detection kits (Abcam, UK).
- Determination of reactive oxygen species (ROS) production assays: MitoSox red dye (Invitrogen, UK) and di-hydrodichlorofluorescein diacetate (CM-H2DCFDA) (Invitrogen, UK).

Preparation of Urtica Urens for Treatments

The leaves of Urtica Urens were obtained from a local market in Jeddah. The leaves were washed, dried, and crushed. Around 50 g of the crushed leaves were soaked in 1.5 L of boiling water for 30 minutes at room temperature for preparing freeze-dried leaves. The mixture while boiling was occasionally stirred. After boiling, the extract was filtrated. The filtrate obtained after filtration was freeze-dried and stored at -20°C.

Cell Culture and Cytotoxicity Analysis of Urtica Urens on A549 Cells

Human (A549) lung cancer cell line was obtained from the immunology laboratory at King Fahad Medical Research Center (KFMRC) at King Abdul-Aziz University (KAU) in Jeddah, Kingdom of Saudi Arabia (KSA). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin, and 1% non-essential

amino acids under standard cell culture conditions (5% CO2 at 37°C). The cell cytotoxicity was assessed through three independent experiments of MTT assay.

Determination of Apoptosis by Annexin-V dye

A549 lung cancer cells were incubated with either 5 or 10 µg/ml of aqueous extract of dried leaves or freeze-dried leaves preparations of Urtica Urens for 24 hours when these cells were 70% confluent in 25 cm^2 flasks. Then, flow cytometry was performed following the manufacture's instructions and 10,000 events in the gated regions were acquired using an emission wavelength of 520 nm for cells labeled with Annexin V-FITC and 620 nm for cells labeled with propidium iodide (PI) on Beckman Coulter flow cytometer.

Detection of Cell Cycle Arrest and DNA Fragmentation

In 25 cm^2 flasks, 70% confluent A549 cells were incubated with 5 or 10 µg/ml of aqueous extract of dried leaves or freeze-dried leaves preparations of Urtica Urens for 24 hours. After that, cells were trypsinized and pellets were washed twice with PBS, fixed with 1 ml of ice-cold fixing buffer (70% ethanol in PBS), and incubated at 4ºC overnight. Then, the cells were incubated for 30 min at 37ºC. Following the incubation, 500 µl PBS followed by 5 µl of 5 mM RNase were added to re-suspend the cells. Five µl of PI (1 mg/ml) was added and cells were examined on BD FACSCalibur flow cytometer to acquire 10,000 events at an emission wavelength of 620 nm, to capture the changes in the cell cycle.

Determination of Cellular and Mitochondrial Response of Cells to Reactive Oxygen Species (ROS)

Di-hydrodichlorofluorescein diacetate (H2-DCFDA) dye and Mitosox red dye assays were performed to assess cellular and mitochondrial ROS levels, respectively in lung A549 cancer cells. For cellular ROS measurement, cells were first cultured in a 96-well plate and then treated with 5 or 10 µg/ml of aqueous extract of freeze-dried leaves preparations of Urtica Urens for 24 hours. After that, 5 µl of DCFDA was added to each well and incubated in the dark for 30 min at 37ºC/ 5% CO2. The resulted DCF fluorescence was read in a BioTek Fluorescence microplate reader at excitation and emission wavelengths of 485 nm and 528 nm, respectively. For the mitochondrial ROS (mtROS) measurements, cells were treated with 5 or 10 µg/ml of aqueous extract of freeze-dried leaves preparations of Urtica Urens under the same culture conditions as cellular ROS experiments. Then, the resulted red fluorescence that represents the level of superoxide produced in the cells was measured with BioTek fluorescence microplate reader according to the manufacture instructions (was read at an excitation wavelength of 530 nm and at an emission wavelength of 590 nm). For each assay, three independent experiments were performed to ensure reproducibility.

Statistical Analysis

Graph Pad Prism software version 6 was used to analyze the data obtained from all assays. The significant differences were evaluated using a one-way analysis of variance (ANOVA) followed by Bonferroni's test correction. A p-value ≤ 0.01 was considered significant. The untreated A549 cells in this study represent the control and it was used to compare the results of all experiments with treated A549 cells.

Results

Cytotoxicity Analysis of Urtica Urens on A549 Cells

The cytotoxic effect of 5 and 10 µg/ml of aqueous extract of dried leaves or freeze-dried leaves preparations of Urtica Urens on lung A549 was assessed by MTT assay. Data revealed that at 10 µg/ml of both aqueous extract of dried leaves and freeze-dried leaves of Urtica Urens has a significant effect on the cell viability of A549 compared to untreated control cells (Figure 1). The cell viability percentage of HCT-116 cells under dried leaves and freeze-dried leaves of Urtica Urens (10 µg/ml) was reduced to 72.18% and 74% compared to untreated HCT-116 (control).

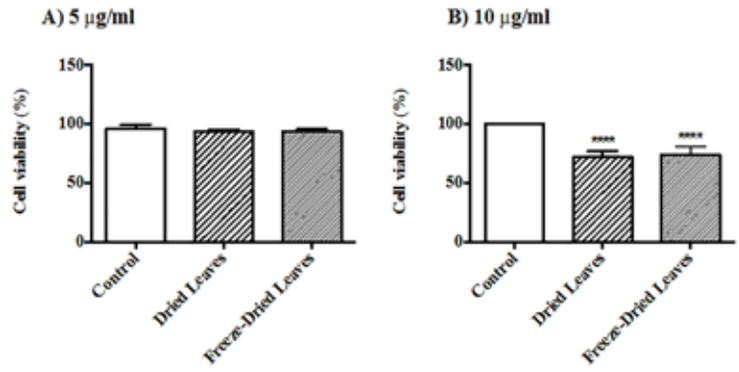

Figure 1. MTT assay of lung A549 cells treated with two concentrations (5 or 10 µg/ml) of aqueous extract of dried leaves or freeze-dried leaves preparations of Urtica Urens for 24 hours.

Determination of Apoptosis by Annexin-V Dye in A549 Cells

Apoptosis (programmed cell death) mechanism and necrosis were investigated in A549 cell line under the treatment of two concentrations (5 & 10 µg/ml) of aqueous preparations of dried leaves and freeze-dried leaves of Urtica Urens. The results showed that treated A549 cells did not respond to both aqueous preparations of Urtica Urens at the early and late phase of apoptosis at 5µg/ml (Figure 2), as compared to the untreated control. Moreover, cells did not go under necrosis process. In contrast, 10 µg/ml of aqueous extract of dried and the freeze-dried Urtica Urens showed a significant rise in late apoptosis phase and necrosis, respectively indicating that A549 cells respond only to high concentration of Urtica Urens.

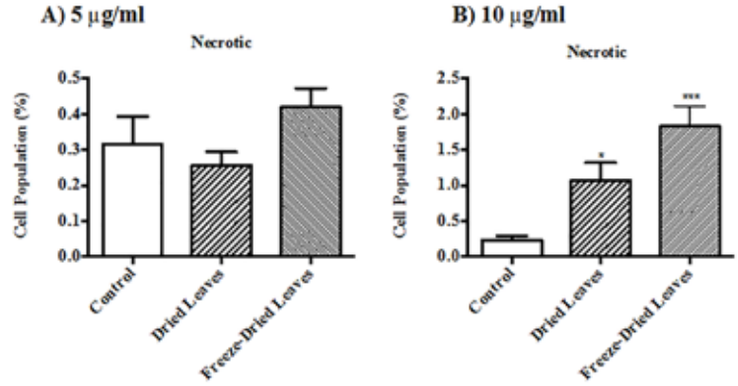

Figure 2. Cell death mechanisms of A549 cells treated with Urtica Urens for 24 hours.

Detection of Cell Cycle Arrest and DNA Fragmentation in A549 Cells

Figure 3 represents the A549 cell cycle phases after treatment with 5 or 10µg/ml of aqueous extracts of dried leaves or freeze-dried leaves of Urtica Urens. Under 5µg/ml concentration, the cell cycle did not show any arrest at any phase for both aqueous extracts of dried leaves or freeze-dried leaves of Urtica Urens. Under 10µg/ml concentration, cells were arresting either in G0/G1 phase (in freeze-dried leaves extract) or in G2/M phase (in dried leaves extract).

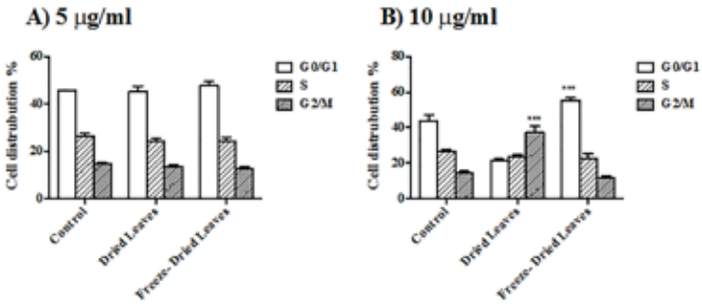

Figure 3. Cell cycle arrest and DNA fragmentation in A549 cells treated with 5 and 10µg/ml of Urtica Urens for 24 hours.

Determination of Cellular and mitochondrial response of A549 cells to Reactive Oxygen Species (ROS)

The calculated cellular or mitochondrial ROS formation intensity showed no significant difference in the cellular or mitochondrial ROS production in lung A549 cells treated with 5 µg/ml compared to the control in both aqueous extracts (dried leaves and freeze-dried) of Urtica Urens (Figure 4). In contrast, cells treated with 10 µg/ml aqueous extract of both aqueous extract of dried leaves and freeze-dried leaves of Urtica Urens was significantly producing more cellular and mitochondrial ROS, as compared to the untreated A549 cells control.

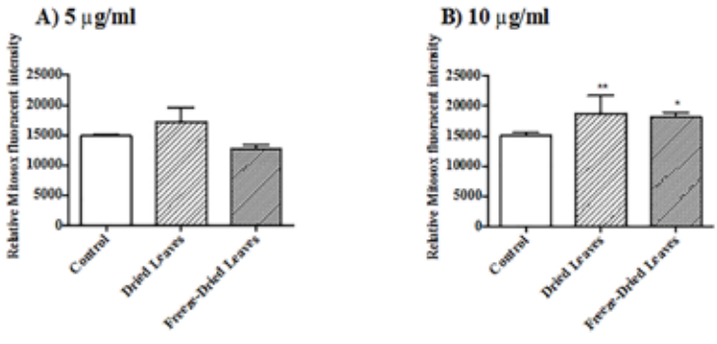

Figure 4. Measurement of cellular and mitochondrial ROS production in HCT-116 cells treated with Urtica Urens for 24 hours.

Discussion

Over the past 30 years, there is a dramatic increase in the use of herbal medicine for treating cancer. These medicines are not only used for treating cancer, rather they are used for reducing the toxicity induced after the use of chemotherapeutic drugs [16].

The present study has illustrated the cytotoxic effect of dried and freeze-dried leaves of Urtica Urens on A549 lung cancer cell lines. The ability of cytotoxic agent to destroy the living cells is termed as cell cytotoxicity. This results in either necrosis or apoptosis of the normal cells. Measurement of cell toxicity holds significant importance as it is an important factor in the development of therapeutic anti-cancer drugs.

The present study conducted MTT experiments to investigate the effect of Urtica Urens on A549 cells tackled with different concentrations for an entire day. Minimum of three independent MTT experiments were conducted, whereas, the outcome was made using one way that has been persuaded by Bonferroni's test. The cell viability was considerably declined in juxtaposition with the untreated cell at 10ug/ml of either the dried leaves or freeze-dried ones of Urtica Urens. It has been noted that the cell death mechanism of the A549 cells that have been dealt with Urtica Urens for 24 hours elucidated that Urtica Urens has no impact on A 549 cells early and late apoptosis or necrosis at 5 µg/ml of both dried and freeze- dried preparation. Whereas, the lung A549 cells retort to the Urtica Urens treatments with 10µg/ml l concentration in late apoptosis (at the dried leaves extract) and necrosis (freeze- dried leaves extract) respectively. The results of the present study revealed that using dried and freeze-dried extracts of Urtica Urens in high concentration significantly reduced the cell viability of the A549 lung cancer cell lines. A similar study conducted by Shofian et al. [16] studied the impact of freeze-dying on the antioxidant activity of different compounds. The results demonstrated that the antioxidant power value of the freeze-dried leaves is significantly low. However, the cell viability was significantly reduced in the three types of cancer cells without resistance in the lung cancer cell line at a high concentration of freeze-dried leaves. These results contradict with the results of the present study. The results stated that freeze-dried Urtica Urens shows a significant difference in the intensity of cellular ROS and mtROS production when they are treated with 10µg/ml.

The values of 3 independent flow Cytometry experiments were considered based on cell cycle arrest and DNA fragmentation in A549 cells treated with 5 and 10µg/ml of Urtica Urens. The functional method followed by the Bonferroni's test namely clarified that the concentration of 5µg/ml did not lead to any arrest. However, at 10µg/ml cells started to arrest either at the phase of G0/G1 at dried leaves extract or at G2/M phase as freeze- dried leaves, respectively. Accordingly, the given results pointed that HCT-116 cells have been treated with the aqueous preparation of dried leaves or freeze-dried Urtica Urens indicating a substantial difference in the intensity of both the cellular ROS and MT ROS production, as they were tackled by 10µg/ml that merely contrast with control the untreated cells.

Like Urtica Urens, Urtica Dioica being a member of Urticaceae family is known to be interchangeable, therapeutically. The cytotoxic activity of Urtica Dioica on cancer cell lines has also

been investigated [17].

For instance, a study conducted by Fattahi et al. [18] showed a significant effect of Urtica Dioica on BT-474 and Hela cell lines. Moreover, another study showed a significant effect of Urtica Dioica on human prostate carcinoma LNCaP cells [17].

In a similar context, few of the previous studies have shown a decrease in the proliferation of MCF-7 cell line after being treated with aqueous extracts of Urtica Dioica leaves. Multiple mechanisms are developed by the cancer cells that help avoid apoptosis and escape the regulated growth of cells [7].

Therefore, it is stated that there is an advantage of using whole-cell extracts containing several components, as compared to an isolated plant product. There is a significant effect of using whole plant extracts in combination with other medications, as plant species like Urtica and Origanum have a long history of oral use.

Conclusion

The present study has investigated the effect of Urtica Urens on A549 lung cancer cell line. The results demonstrated that 10 µg/ml of both aqueous extract of dried leaves and freeze-dried leaves of Urtica Urens had a significant effect on the cell viability of A549 compared to untreated control cells. It was indicated that A549 cells respond only to a high concentration of Urtica Urens as 10 µg/ml of dried and the freeze-dried Urtica Urens showed a significant rise in late apoptosis phase and necrosis, respectively. Moreover, Urtica Urens was significantly producing more cellular and mitochondrial ROS, as compared to the untreated A549 cells control. The study concluded that freeze-dried extracts of Urtica Urens leaves have cytotoxic effects on cancer cell lines, under certain concentrations. Additional in vivo studies on animal models and human clinical trials are needed for further testing of human specific use of these plants for treating cancer cell lines.

Acknowledgment

The authors are thankful to all the associated personnel who contributed for this research.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

Funding: *None*

Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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How to cite this article:

Ulfat Omar, Akram Aloqbi. The effect of Urtica Urens on A549 lung cancer cell line. Ann Clin Anal Med 2020;11(5):510-514